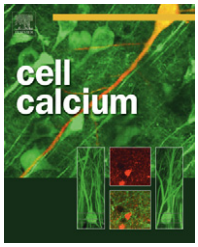
Cell Calcium (2008) 44, 298–309

journal homepage: www.elsevier.com/locate/ceca



Ca^{2+} signaling in injured *in situ* endothelium of rat aorta

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Received 19 May 2007; received in revised form 20 November 2007; accepted 17 December 2007
Available online 13 February 2008

KEYWORDS
In situ endothelium;
Rat aorta;
Injury;
Ca^{2+};
ATP;
Gap junctions

Summary The inner wall of excised rat aorta was scraped by a microelectrode and Ca^{2+} signals were investigated by fluorescence microscopy in endothelial cells (ECs) directly coupled with injured cells. The injury caused an immediate increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), followed by a long-lasting decay phase due to Ca^{2+} influx from extracellular space. The immediate response was mainly due to activation of purinergic receptors, as shown by the effect of P_{2X} and P_{2Y} receptors agonists and antagonists, such as suramin, α,β-MeATP, MRS-2179 and 2-MeSAMP. Inhibition of store-operated Ca^{2+} influx did not affect either the peak response or the decay phase. Furthermore, the latter was: (i) insensitive to phospholipase C inhibition, (ii) sensitive to the gap junction blockers, palmitoleic acid, heptanol, octanol and oleamide, and (iii) sensitive to La^{3+} and Ni^{2+}, but not to Gd^{3+}. Finally, ethidium bromide or Lucifer Yellow did not enter ECs facing the scraped area.

These results suggest that endothelium scraping: (i) causes a short-lasting stimulation of healthy ECs by extracellular nucleotides released from damaged cells and (ii) uncouples the hemichannels of the ECs facing the injury site; these hemichannels do not fully close and allow a long-lasting Ca^{2+} entry.

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0143-4160/$ – see front matter
doi:10.1016/j.ceca.2007.12.007

1. Introduction

The process of wound repair is an intricate biological response that appears to be triggered by an injury-evoked signal(s) affecting the cells located near the edge of the damage. The activation and coordinate interaction of multiple signaling pathways then occur, eventually leading to wound healing [1]. The triggering signal(s) might be the removal of contact inhibition mediated by cell surface proteins [2,3], the extracellular release of paracrine stimulatory signals from injured cells and a transient increase in intracellular Ca2$^+$ concentration ($[Ca^{2+}]_i$) [4–8]. The subsequent processes of cell migration and proliferation require the involvement of growth factors, such as epidermal growth factor in epithelial cells [9], basic fibroblast growth factor and vascular endothelial growth factor in endothelial cells (ECs) [10–12], and platelet-derived growth factor, which has been shown to favor ulcer healing in diabetic patients [13].

When the endothelium is injured, an increase in $[Ca^{2+}]_i$ is an immediate response of the cells near the injury site. The Ca^{2+} signal, which initiates in the site of injury and quickly propagates to neighboring cells, consists of a fast peak response which decreases to a plateau level after 1 min and returns to the baseline within 8 min [14,15]. The increase in $[Ca^{2+}]_i$ was found to be sensitive to removal of extracellular Ca^{2+} [4,5,14], depletion of internal Ca^{2+} stores [4,5,7] and nucleotide antagonists [4,6]. In ECs, Ca^{2+} signals can be evoked by a number of mechanisms. Many agonists, such as nucleotides, acetylcholine (Ach) and growth factors, are able to trigger phospholipase C (PLC) activation, inositol 1,4,5-trisphosphate (IP_3) production and Ca^{2+} release from the endoplasmic reticulum. In the absence of Ca^{2+} influx from the extracellular medium, a short-duration $[Ca^{2+}]_i$ increase occurs [16]. The presence in ECs of a Ca^{2+}-induced Ca^{2+} release mechanism mediated by ryanodine-receptors has also been proposed, but evidence about its importance is limited [17,18]. IP_3-store depletion results in the opening of Ca^{2+}-permeable channels on the plasma membrane, the so-called store-operated channels (SOCs), which prolong the Ca^{2+} signal and refill the stores [19]. This mode of Ca^{2+} inflow, which depends on the physical coupling between the STIM protein on the Ca^{2+} store and the plasma membrane Ca^{2+} channels (either TRPC1 or Orai) [20,21], is the predominant pathway of Ca^{2+} entry in non-excitable cells, including ECs [19,22–24]. Additional routes for Ca^{2+} influx may be provided by: (1) receptor-activated cation channels (RACCs), activated by intracellular second messengers, such as diacylglicerol [19,25]; (2) mechanosensitive Ca^{2+}-permeable channels (MCC), activated by stretch, pressure and shear stress [19]; (3) L- and T-like voltage-dependent Ca^{2+} channels (VGCC), whose expression in ECs is, however, rather scarce [19,26].

Previous investigations on Ca^{2+} signals in injured endothelium employed cell culture models and made no distinction between peak response and decay phase. In this study, the mechanisms inducing the peak response and the subsequent long-lasting decay phase were separately investigated. Furthermore, the experiments were performed on *in situ* ECs of excised rat aorta, a more relevant physiological condition than cultured systems [16].

2. Methods

2.1. Dissection of the aorta

Wistar rats aged 1–3 months were sacrificed with an overdose of diethyl ether. The thoracic and abdominal aorta were dissected out and perfused with physiological salt solution (PSS). The vessel was cleaned of the surrounding connective tissue, cut in ~5 mm long rings, stored in PSS at room temperature (22–24 °C) and used within 5 h.

2.2. Solutions

PSS had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes. In Ca^{2+}-free solution, Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Solutions were titrated to pH 7.4 with NaOH. Aortic rings were bathed in Ca^{2+}-free solution for no longer than 90 s min before inducing the injury. Control experiments were performed to assess whether such a short pre-incubation was able to deplete intracellular Ca^{2+} stores. The amplitude of the response to the $InsP_3$ mobilizing agonist ATP did not significantly change in cells incubated in Ca^{2+}-free solution for less than 30 s (the shortest time required to fully change the solutions in the bath) and 90 s, respectively (0.105 ± 0.0070, $n = 65$, and 0.0967 ± 0.0069, $n = 61$; $p = 0.43$).

2.3. $[Ca^{2+}]_i$ measurements

The technique used to evaluate changes in $[Ca^{2+}]_i$ in intact endothelium has been previously described [27]. Briefly, the aortic ring was opened and loaded with 16 μmol/L fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. *In situ* ECs were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Germany), usually equipped with a Zeiss 63× Achroplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). ECs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the exciting light and a second neutral density filter (optical density = 0.3) was coupled to the 380-nm filter to approach the intensity of the 340-nm light. A round diaphragm was used to increase the contrast. The exciting filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot on-line the fluorescence from 10 to 15 rectangular ''regions of interest'' (ROI) enclosing 10–15 single cells (see Fig. 1A). Each ROI was identified by a number. Since cell borders were not clearly identifiable, a ROI may not include the whole EC or may include part of an adjacent EC. Adjacent ROIs never superimposed. $[Ca^{2+}]_i$ was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly called ''ratio''). An increase in $[Ca^{2+}]_i$ causes an increase in the ratio [27]. Ratio measurements

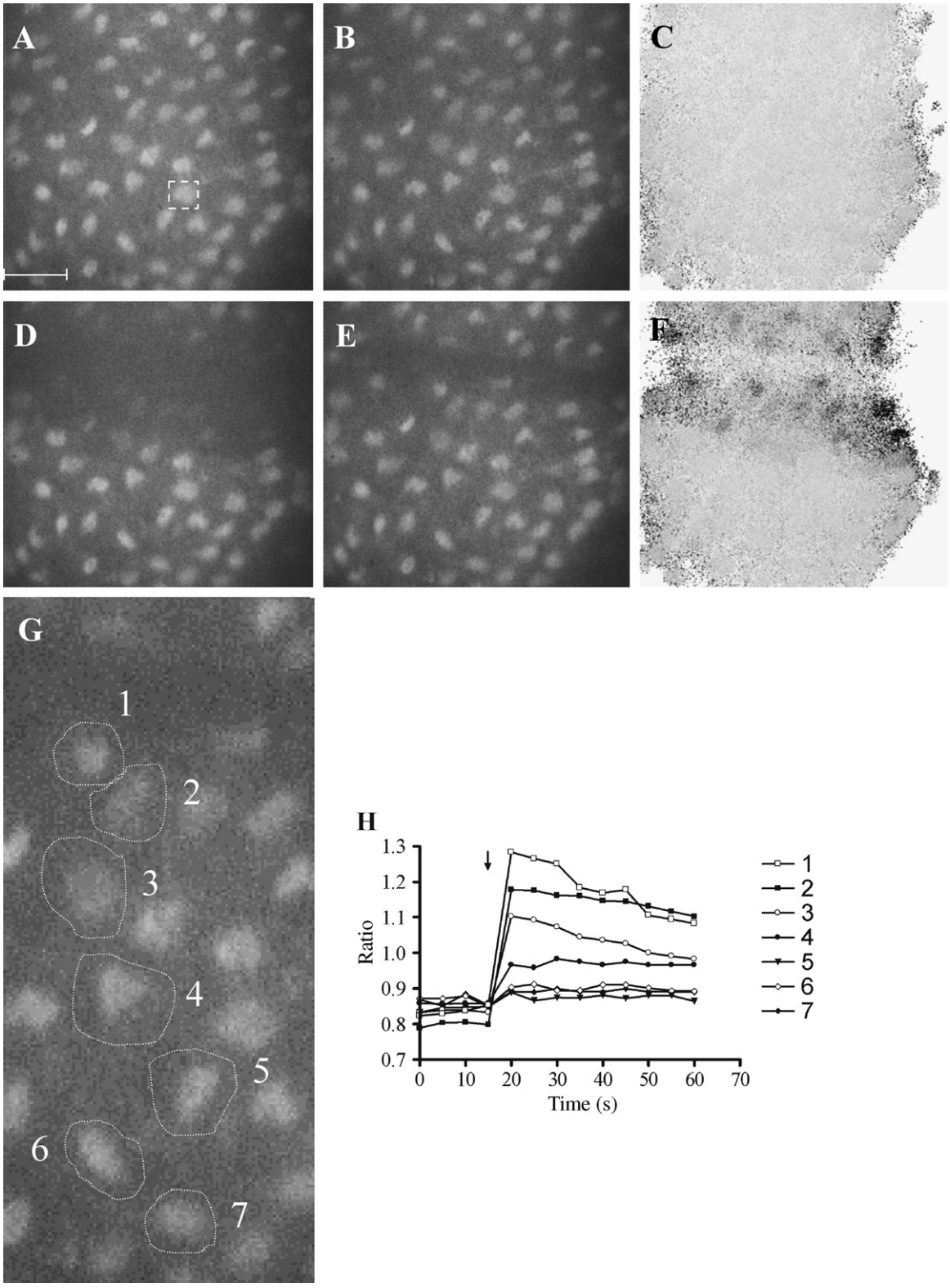

Figure 1 $[Ca^{2+}]_i$ increase induced in ECs by damaging rat aorta inner wall. (A) An intact patch of endothelium loaded with fura-2 and excited at 380 nm. An example of rectangular ROI has been drawn. The bar length is 40 μm. (B) The same patch of panel A excited at 340 nm. (C) The ratio between B and A (B/A), the darker the color the greater the $[Ca^{2+}]_i$. (D and E) The same patch after the lesion, excited at 380 nm (D) and 340 nm (E). (F) The ratio E/D, the darker the color the greater the $[Ca^{2+}]_i$. (G) An enlarged part of E with seven ROIs of arbitrary shape located at increasing distances from the injury. (H) The fluorescence ratios measured in these seven ROIs are reported. In this and the following figures, the arrow indicates when injury is performed. Scion Image software has been here employed.

were performed and plotted on-line every 3—5 s. Off-line analysis could also be performed by recording images of the entire field of cells and using custom-made macros developed by Scion Corporation software (www.scioncorp.com). The experiments were performed at room temperature.

2.4. Mechanical disruption of ECs

ECs were injured under microscopic control by means of a glass microelectrode with a broken tip of about 30 μm diameter, driven by an XYZ hydraulic micromanipulator (Narishige, Japan). The microelectrode was first positioned almost parallel and very near to the endothelium surface. It was then moved downward, along the *Z*-axis, until the electrode tip slightly touched the endothelium, and moved horizontally across the visual field. This procedure allowed monitoring of Ca^{2+} signals during injury. Images of fura-2-loaded ECs, together with numbered ROIs, were taken before and after the injury, in order to identify ECs adjacent to the injury.

2.5. ECs viability

Ethidium bromide, a fluorescent molecule unable to cross an intact plasma membrane and therefore indicative of damaged/dead cells, was used to check ECs viability.

2.6. Chemicals

Fura-2/AM and Lucifer Yellow were obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). *N*-(4-[3,5-Bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP-2) was purchased from Calbiochem (La Jolla, CA, USA). All other chemicals were from Sigma.

2.7. Data analysis

For each protocol, data were collected from at least three rats. The amplitude of the peak response was measured as the difference between the ratio at the peak and the mean ratio of 1-min baseline before the peak. Such a difference was considered as a physiological signal when it was >2 times the SD of the baseline. Mean values are presented together with standard error of the mean and the number n of tested cells. Statistical comparisons of peak amplitudes were made by Student's *t*-test. As to the decay phase, the number *x* of cells responding to the experimental test over the number *y* of tested cells (*x*/*y*) is usually reported. Unless differently stated, tracings shown in the figures are single ROI recordings.

3. Results

3.1. Preliminary observations

Under microscopic observation, the inner wall of the aortic rings often showed patches fully covered by ECs (Fig. 1A and B). ECs not intentionally damaged, probably during the dissection procedure, might be present, as evidenced by applying ethidium bromide (not shown). Cells that incorporated ethidium bromide did not emit fura-2 fluorescence and, conversely, cells showing fura-2 fluorescence excluded ethidium bromide (not shown). Before damaging the endothelium, ECs integrity was checked by looking at the presence of a continuous layer of fura-2-loaded cells.

The microelectrode scraped one to two rows of cells (Fig. 1D and E) and an evident increase in the $[Ca^{2+}]_i$ occurred in the cells near the lesion (Fig. 1F and H). The increase in $[Ca^{2+}]_i$ was less remarkable with increasing distance from the injured site. Beyond three to four rows of cells away from the lesion, no evident $[Ca^{2+}]_i$ elevation could be observed (Fig. 1H, cells # 5, 6, and 7).

In the subsequent part of this investigation only Ca^{2+} signals occurring in ECs adjacent to the injured area, which have partially lost the contact with neighboring ECs, will be considered for analysis (for example, in Fig. 1G, cell #1). In the large majority (365/380) of these cells, the Ca^{2+} transient induced by mechanical injury consisted in a rapid increase in $[Ca^{2+}]_i$ (the peak response: 0.21 ± 0.0047, $n = 231$), followed by a long-lasting decay toward the original baseline (Fig. 2A). Cells exhibiting such a long $[Ca^{2+}]_i$ increase still responded to extracellular ATP during the decay, suggesting that Ca^{2+} homeostasis was conserved (Fig. 2A; 63/68). In a fraction of cells (16% of responding cells), Ca^{2+} oscillations superimposed the decay phase of the Ca^{2+} transient (Fig. 2B). The mechanism sustaining the oscillations was not investigated. Endothelium injury caused an immediate increase in $[Ca^{2+}]_i$ also in the absence of extracellular Ca^{2+} in 76 out of 98 cells, although the prolonged decay phase disappeared (Fig. 2C). In addition, the $[Ca^{2+}]_i$ increase observed in Ca^{2+}-free solution was significantly smaller than the value measured in presence of extracellular Ca^{2+} (0.12 ± 0.0049, $n = 142$; $p < 0.01$) (Figs. 2C and 8A). The decrease in the magnitude of the initial Ca^{2+} raise was not due to Ca^{2+} pool depletion (see Section 2). These results indicate that the peak response is thus due to both Ca^{2+} influx and Ca^{2+} release, whereas the decay phase is mediated only by Ca^{2+} inflow.

3.2. The peak response: the predominant role of purinoreceptors

ATP is released from injured large vessels ECs and can reach high concentrations (50—100 μM) in proximity of the surface of ECs facing the wound edge [28]. ATP, or metabolites deriving from ectoenzymatic breakdown [27], may, in turn, increase the endothelial $[Ca^{2+}]_i$ via stimulation of the ionotropic Ca^{2+}-permeable P_{2X} receptors and the G-protein coupled P_{2Y} receptors [29]. Thus, the Ca^{2+} increase we observed in rat aortic ECs might be due to ATP liberated from disrupted cells [5]. Consistent with this hypothesis, suramin (300 μM), a broad spectrum antagonist of P_2 receptors [29], decreased by 79.2% the amplitude of injury-elicited Ca^{2+} response (Fig. 8A). In order to dissect out the contribution of P_{2X} and P_{2Y} receptors, we used a number of agonists and antagonists of P_2 receptors. The putative P_{2X} receptors agonist, α,β-MeATP (3 μM) [30], induced a transient increase in $[Ca^{2+}]_i$ (0.065 ± 0.009, $n = 23$), which disappeared upon removal of extracellular Ca^{2+} (not shown), in agreement with

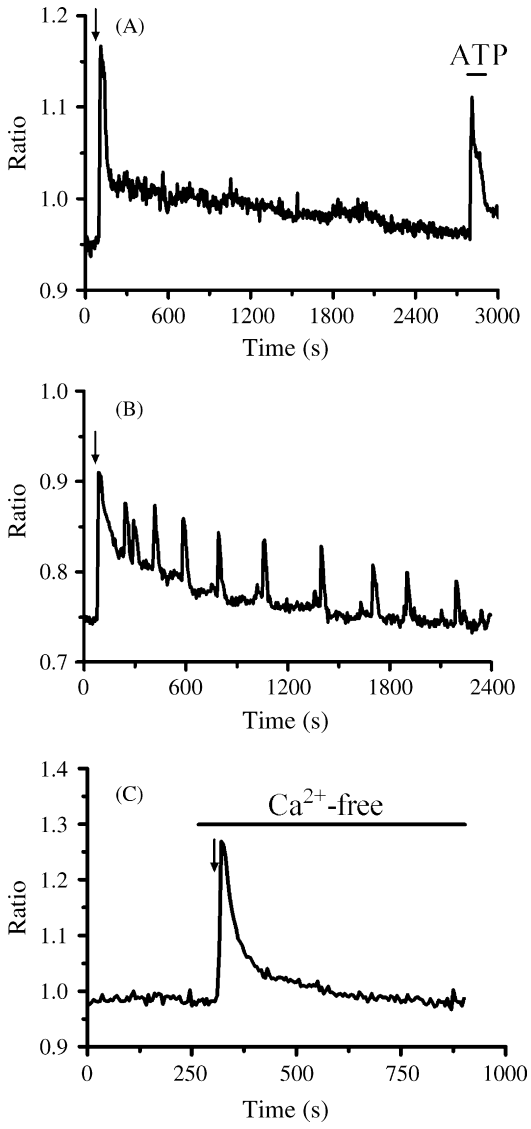

Figure 2 Damaging rat aorta inner wall evokes long-lasting Ca^{2+} signals in ECs facing the injury. (A) Prolonged increase in $[Ca^{2+}]_i$ induced by lesioning rat aorta in Ca^{2+}-containing solution. ATP (20 μM) has been applied in the late part of the decay phase. (B) Ca^{2+} oscillations may overlay the decay phase. (C) The Ca^{2+} elevation is significantly shorter in absence of extracellular Ca^{2+}.

the expected activation of a Ca^{2+}-permeable channel [29]. When α,β-MeATP (3 μM) was re-added 1 min after the first application, no Ca^{2+} transient was recorded (not shown), due to the reported desensitization of P_{2X} receptors [30]. Notably, desensitizing P_{2X} receptors with α,β-MeATP (3 μM) reduced by ≈15% the height of injury-induced rise in $[Ca^{2+}]_i$ (Fig. 8A), suggesting that the P_2-dependent component of the signal is mainly operated by P_{2Y} receptors.

The dominant P_{2Y} receptor subtypes present in vascular endothelial cells are P_{2Y1} and P_{2Y2} [31], a feature which applies also to rat aortic endothelial cells [27,32; see Discussion]. In addition, it has recently been shown that rat brain capillary ECs may express a P_{2Y12} receptor [33]. In absence of specific inhibitors of P_{2Y2} receptors [31], we assessed the effect of MRS-2179 and 2-MeSAMP, which antagonize P_{2Y1} and $P_{2Y12/13}$ receptors, respectively, on the Ca^{2+} response to endothelial damage. As shown in Fig. 8A, both drugs caused a modest, but significant, inhibition of the Ca^{2+} peak amplitude. Taken together, these data demonstrate that: (1) ≈80% of the magnitude of injury-induced Ca^{2+} increase is mediated by P_2 receptors, likely activated by extracellularly released ATP or ATP-derived nucleotides [27; see Discussion] and (2) P_{2X} receptors mediate only ≈15% of the injury-induced Ca^{2+} increase, the 65% of which is mainly attributable to P_{2Y2} receptors, with a minor aid by P_{2Y1} and $P_{2Y12/13}$ receptors.

3.3. The peak response: P_{2Y} receptors induce Ca^{2+} release from intracellular stores

P_{2Y} receptors are known to mobilize intracellular Ca^{2+} from IP_3-sensitive stores upon PLC activation and to stimulate extracellular Ca^{2+} entry via SOCs [31]. As previously shown, the peak response to injury is due to both Ca^{2+} influx and Ca^{2+} release. In order to elucidate the intracellular signaling pathway(s) recruited by P_{2Y} receptors following endothelial damage, ECs were scratched in presence of suramin (300 μM) after removal of extracellular Ca^{2+}. The application of suramin in Ca^{2+}-free solution fully inhibited any injury-induced increase in $[Ca^{2+}]_i$ (Fig. 3A; $n = 33$), thus suggesting that P_{2Y} receptor activation induces Ca^{2+} release from an intracellular pool. Suramin is a fluorescent molecule and its application causes an immediate reduction in the basal fluorescence ratio (Fig. 3A). However, the presence of the drug still allows the fluorimetric system to monitor $[Ca^{2+}]_i$ increase, as indicated by stimulating the ECs with Ach after suramin addition (Fig. 3B; $n = 18$). Incubation with U73122 (50 μM), a widely employed PLC inhibitor [34], in Ca^{2+}-free solution reduced the peak amplitude of injury-induced Ca^{2+} transient to 0.055 ± 0.022, which is significantly smaller than the value measured only in the absence of extracellular Ca^{2+} (Fig. 3C; $n = 37$; $p < 0.05$). This result, which agrees with the inhibitory effect of suramin in Ca^{2+}-free-solution, suggests that Ca^{2+} liberation from IP_3 receptors (IP_3Rs) contribute to the onset of P_{2Y} receptors-mediated Ca^{2+} response to injury, although U73122 may affect other forms of Ca^{2+} signaling [35–37].

In order to understand whether SOCs activation did sustain P_{2Y} receptor-promoted $[Ca^{2+}]_i$ elevation, we first tested the inhibitory effect of BTP-2, a recently discovered selective inhibitor of these channels [38,39], on ATP- and cyclopiazonic acid (CPA)-induced Ca^{2+} inflow. CPA (10 μM), an inhibitor of the endoplasmic reticulum (ER) Ca^{2+}-ATPase, prevents Ca^{2+} reuptake into the stores, thus leading to their depletion and to SOCs activation [40]. In Ca^{2+}-free solution, CPA evoked a transient increase in $[Ca^{2+}]_i$ due to passive emptying of Ca^{2+} stores through ER leak channels (Fig. 4A). Extracellular Ca^{2+} was then readded (the classical Ca^{2+} addback protocol) and the amplitude of Ca^{2+} influx measured. Fig. 4A clearly shows that store-operated Ca^{2+} influx was strongly inhibited by BTP-2 (20 μM) in 79 out of 79 cells. To investigate the involvement of SOCs in ATP-induced Ca^{2+} inflow, the cells were stimulated with a concentration of the nucleotide able to activate Ca^{2+} influx [27]. As depicted in Fig. 4B, BTP-2 (20 μM) fully blocked ATP-induced Ca^{2+} entry, by decreasing the peak response to 300 μM ATP

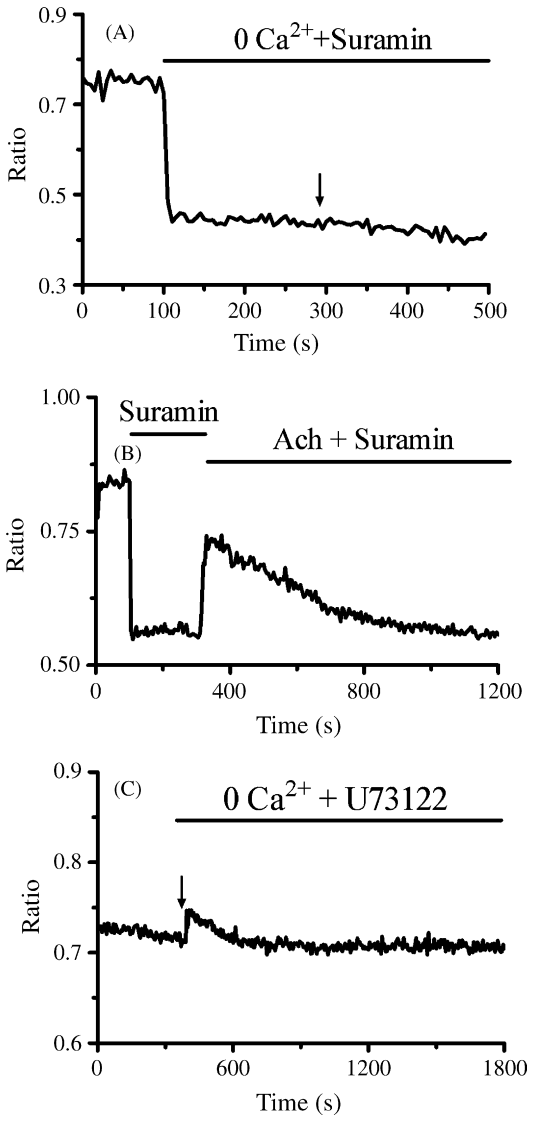

Figure 3 Effect of suramin on injury-induced Ca^{2+} elevation. (A) Null response to injury in the presence of suramin (300 μM) and in the absence of extracellular Ca^{2+}. Note that suramin application causes a quick decrease in fluorescence ratio (see text). (B) Ca^{2+} transient elicited by Ach (10 μM) application in presence of suramin (300 μM). (C) Ca^{2+} transient elicited by injury in the presence of U73122 (50 μM) and in the absence of extracellular Ca^{2+}.

and causing a fast decline of the $[Ca^{2+}]_i$ to the baseline in 32 out of 32 cells. These preliminary observations suggest that BTP-2 is a reliable blocker of SOCs in rat aortic ECs *in situ*, and that this pathway sustains the ATP-dependent Ca^{2+} influx. Subsequently, we assessed the effect of SOCs inhibition on the Ca^{2+} response to endothelial damage. Surprisingly, BTP-2 (20 μM) did not significantly affect either the magnitude of the initial Ca^{2+} raise or the subsequent decay phase (Figs. 4C, 8A and B). In according with this observation, Gd^{3+} (10 μM), a non-selective SOCs blocker [19], did not impair the decay phase of the Ca^{2+} signal (Fig. 4D; 63/63). Collectively, these findings indicate that P_{2Y} receptors mediate injury-dependent Ca^{2+} signaling by inducing Ca^{2+} release from IP_3Rs, but not Ca^{2+} influx. Moreover, P_{2Y} receptors contribute only to the peak Ca^{2+} response to damage, but not to the decay phase.

The data concerning the amplitude of the immediate response upon the aforementioned pharmacological treatments are summarized in Fig. 8A.

3.4. The decay phase: putative role of gap junctions

As previously shown, the prolonged decay phase of injury-evoked Ca^{2+} signal does not occur in Ca^{2+}-free solution (Fig. 2C; $n = 76$). Accordingly, removal of extracellular Ca^{2+} or application of a high-Ca^{2+} extracellular solution (7.5 mmol/L) during the recovery towards the baseline, caused an immediate decrease (Fig. 5A; 62/62) or increase in $[Ca^{2+}]_i$ (Fig. 5B; 41/41), respectively, confirming that Ca^{2+} influx underpins the decay phase. The results obtained with BTP-2 and Gd^{3+} demonstrate that SOCs do not provide the route for such Ca^{2+} entry. Furthermore, PLC inhibition by U73122 (50 μM) had no effect on the decay phase of injury-evoked Ca^{2+} signal (not shown; $n = 42$), another indication that it was consequent neither to IP_3-dependent store depletion nor to Ca^{2+} influx evoked by PLC-derived intracellular second messengers.

ECs seldom express voltage-operated Ca^{2+} channels (VGCC) [19,26], which share some similarities with the classical L- and T-type Ca^{2+} channels [19]. However, depolarizing ECs by an increase in the extracellular concentration of KCl did not elicit any detectable Ca^{2+} transient (data not shown). Consistent with this result, neither Gd^{3+} (see above), a non specific blocker of T-type channels [41], nor verapamil (20 μM), an inhibitor of L-type channels [42] (not shown; 41/41), had any effect on the injury-evoked $[Ca^{2+}]_i$ increase. All together, the above data show that, during the decay phase of the injury-induced Ca^{2+} signal, Ca^{2+} influx occurs, but neither SOCs nor VGCC are involved.

Gap junctions are abundantly expressed in rat aorta ECs [43]. It may be supposed that ECs adjacent to the lesion have gap junction channels that are no longer coupled to other ECs and are exposed to the high-Ca^{2+} extracellular medium. If these channels do not fully close, Ca^{2+} influx can take place. We tested this hypothesis by using gap junction blockers [7,44–46]. Incubating the aortic rings in the presence of 4 mM octanol ($n = 48$) and 200 μM oleamide ($n = 32$) reduced the peak Ca^{2+} response by ≈20% and almost abolished the prolonged decay phase (Figs. 5C and D and 8A). The latter was also strongly reduced by acute application of the drugs in 45 out of 56 cells (Figs. 6E and 8B; octanol) and in 41 out of 53 cells (Figs. 6F and 8B; oleamide), respectively. Consistently, palmitoleic acid (50 μM) and heptanol (4 mM) decreased the $[Ca^{2+}]_i$ in 91% (52/57) and 74% (56/76) of tested cells, respectively, both in the early and in the late part of the decay phase (Fig. 6A–D). The data regarding the percentage of inhibition of the decay phase upon the aforementioned pharmacological treatments are summarized in Fig. 8B. The injury-evoked $[Ca^{2+}]_i$ increase was also sensitive to La^{3+} (5 μM; 31/41), which has been shown to block hemichannels expressed in HeLa cells [47], and Ni^{2+} (50 μM; 30/32) (Fig. 7A and B). In the presence of Ni^{2+}, heptanol application had no effect (Fig. 7B) and, conversely, after heptanol application, Ni^{2+} was without consequence

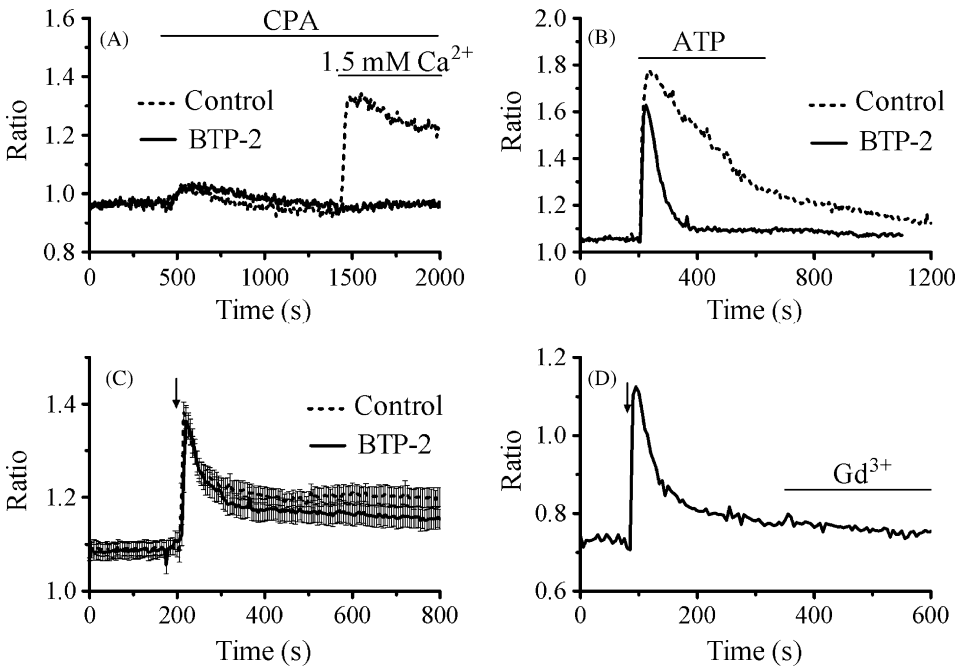

Figure 4 Store-operated Ca^{2+} influx does not mediate the decay phase of injury-evoked Ca^{2+} elevation. (A) Ca^{2+} stores were first depleted with CPA (10 μM) in Ca^{2+}-free solution. Restoration of extracellular Ca^{2+} resulted in store-operated Ca^{2+} entry under control conditions (dashed line). Incubating the aortic rings with BTP-2 (20 μM) for 20 min dramatically decreased CPA-induced Ca^{2+} entry (continuous trace). (B) The amplitude and duration of the Ca^{2+} response to ATP (300 μM) were significantly reduced by pre-exposing aortic rings to BTP-2 (20 μM) for 20 min. (C) Pre-incubation with BTP-2 (20 μM) for 20 min does not affect the Ca^{2+} response to damage. Tracings are the mean ± S.E. of 18 control cells and 25 BTP-2 treated cells, respectively. (D) acute addition of 10 μM Gd^{3+} did not impair the decay phase of the $[Ca^{2+}]_i$ increase evoked by injury.

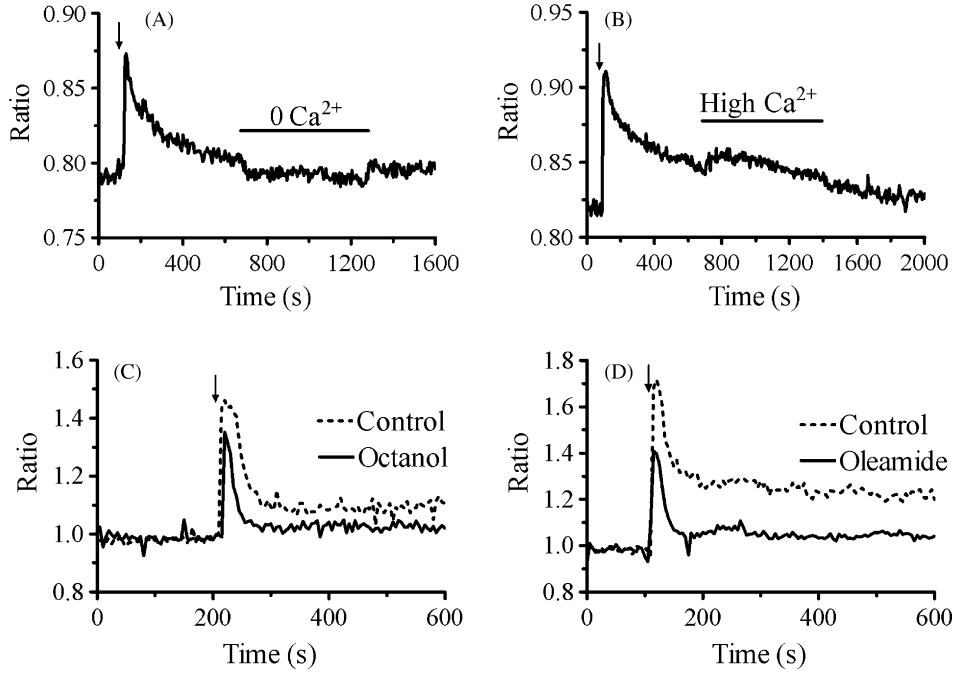

Figure 5 Effect of gap junction blockers on the injury-evoked Ca^{2+} elevation. Effect of removing extracellular Ca^{2+} (A) or applying a high-Ca^{2+} (7.5 mM) extracellular solution (B) on the decay phase of injury-induced increase in $[Ca^{2+}]_i$. Pre-incubation with octanol (4 mM) (C) and oleamide (200 μM) (D) inhibited both the magnitude of the immediate increase and the decay phase.

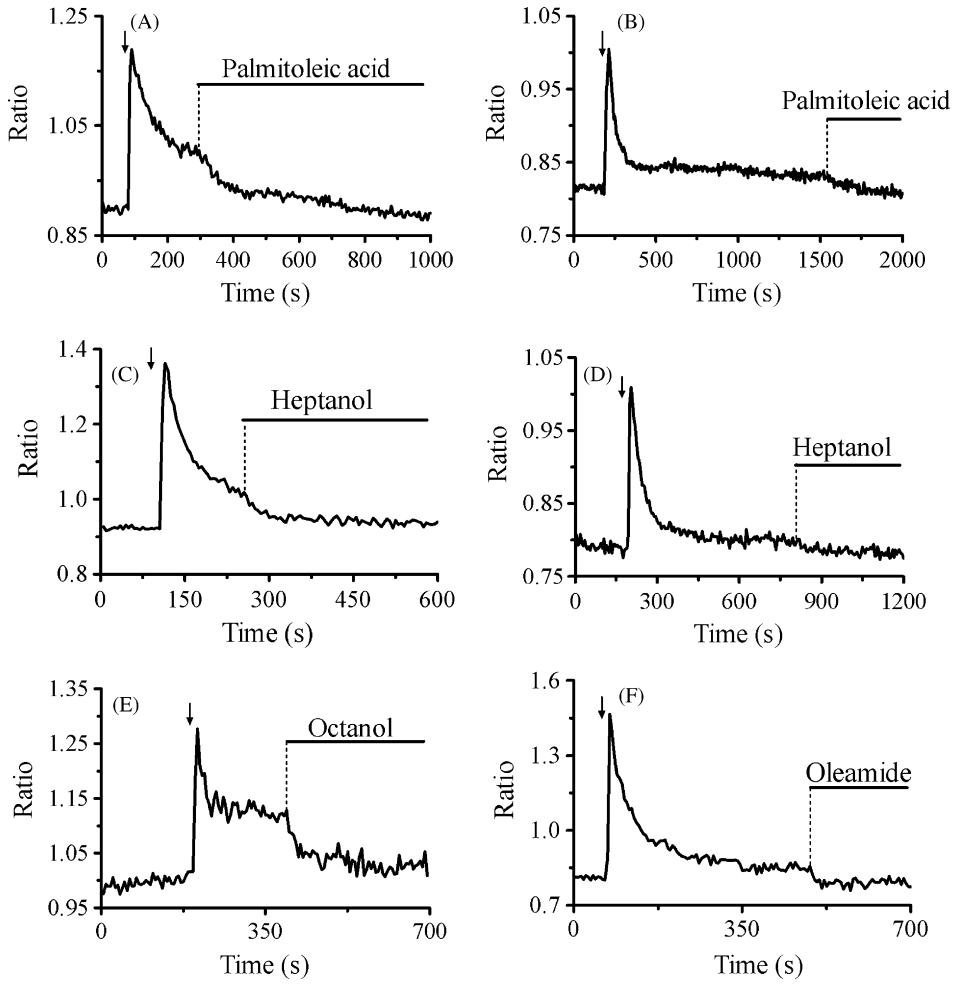

Figure 6 Effect of acute application of gap junction blockers on the decay phase of the injury-evoked Ca^{2+} elevation. Effect of palmitoleic acid (50 μM) on the early (A) and late (B) decay phase of the Ca^{2+} increase. Effect of heptanol (4 mM) on the early (C) and late (D) decay phase of the Ca^{2+} increase. Effect of octanol (4 mM) (E) and oleamide (200 μM) (F) on the late phase of the decay.

(not shown). This suggests that both molecules act on the same Ca^{2+} pathway, likely the gap junctions. It is worth noting that: (1) low micromolar doses of La^{3+} may occlude SOCs [48], however, SOCs are not activated upon endothelial injury; and (2) Ni^{2+} may block also VGCC [41], however, the lack of response to high KCl indicates that the inhibitory action of Ni^{2+} is independent on VGCC.

Notably, in a number of experiments, injury was made in the presence of ethidium bromide and Lucifer Yellow, two molecules able to permeate gap junctions [49,50]. Cells showing the injury-evoked Ca^{2+} transient did not incorporate these dyes (not shown; $n = 35$).

4. Discussion

Regulation of endothelial cell migration and proliferation is central to the process of endothelial regeneration which occurs after a lesion, such as a ballooning injury [51]. Previous studies performed on monolayers of ECs in culture have shown that an increase in $[Ca^{2+}]_i$ plays a key role in stimulating endothelial growth and movement during healing [14,52]. For instance, the $[Ca^{2+}]_i$ elevation may activate the transcription of a number of immediate early genes, which are required for the induction of normal rates of cell motility after wounding [53]. Moreover, an increase in $[Ca^{2+}]_i$ may lead to nitric oxide (NO) production, which in turn promotes ECs proliferation during wound repair [54]. In the present study, we analyzed for the first time the Ca^{2+} signals elicited by tissue injury in ECs on rat aorta *in situ*, a more relevant model in comparison to ECs culture.

The Ca^{2+} signal generated in ECs by damage of rat aorta did not propagate farther than the fourth to fifth row of cells away from the lesion site and consisted in an initial transient peak followed by a prolonged decay phase. The source of the $[Ca^{2+}]_i$ increase was both intra- and extra-cellular. Accordingly, in absence of extracellular Ca^{2+}, the magnitude of the initial Ca^{2+} raise was reduced and the decay phase absent. The involvement of Ca^{2+} release and Ca^{2+} entry in the onset and the propagation of injury-induced Ca^{2+} movements have also been described in CPAE and cultured rat alveolar type II cells [4,14]. However, the signaling pathway shaping the raise in $[Ca^{2+}]_i$ after tissue damage was not fully addressed in these studies [4,14,52].

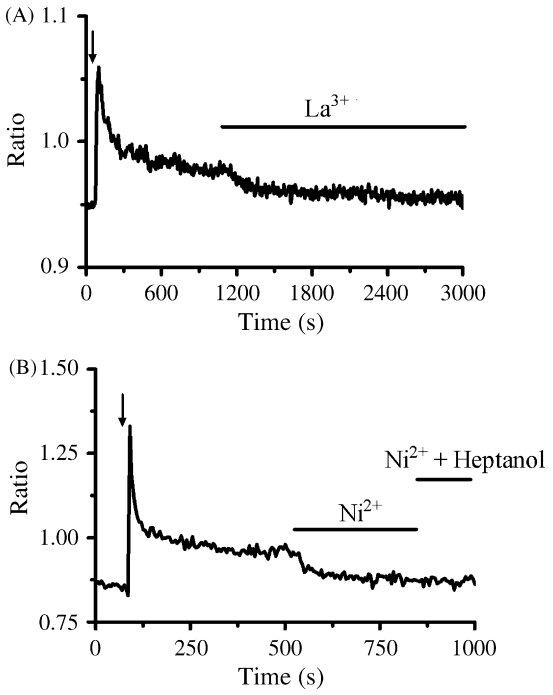

Figure 7 Effect of La^{3+}, Ni^{2+} and heptanol plus Ni^{2+} on the decay phase of the injury-evoked $[Ca^{2+}]_i$ increase. (A) Effect of La^{3+} (5 μM) on the decay phase of injury-evoked Ca^{2+} elevation. (B) Effect of Ni^{2+} (50 μM), and heptanol (4 mM) in the presence of Ni^{2+} (50 μM), on the decay phase of injury-evoked Ca^{2+} elevation.

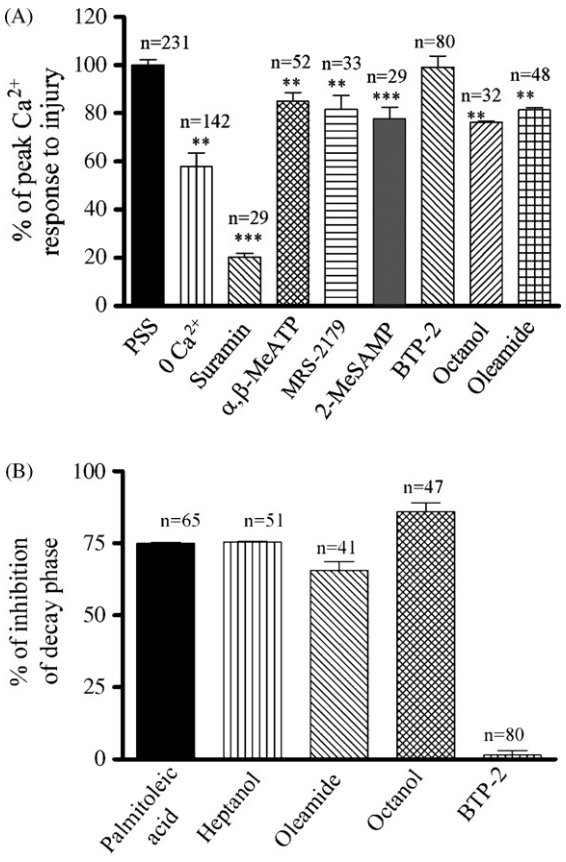

Figure 8 Decrease in the amplitude of the immediate increase in $[Ca^{2+}]_i$ and of the decay phase of the Ca^{2+} signal elicited by damage following the designated treatments. (A) Percentage amplitude of the peak response to injury in presence of designated drugs as compared to control conditions (PSS). Double asterisk indicates a level of significant difference <0.01 and triple asterisks <0.005. (B) Percentage of inhibition of the decay phase of injury-evoked Ca^{2+} increase following acute application of gap junctions blockers and pre-incubation with BTP-2. The number of ECs analyzed in each condition is indicated above the bars. See the text for drugs concentrations.

4.1. The injury-evoked Ca^{2+} signal: the peak

Significant amounts (50—100 μM) of ATP may transiently accumulate at injury sites following release from lesioned endothelial and smooth muscle cells, which contain up to 1 mM ATP [28]. Additional amounts of this nucleotide are liberated from platelets and mast cells recruited to the sites of tissue damage [55]. Furthermore, ATP might be released via hemichannels by ECs adjacent to the injury, as shown in mechanically stimulated cultured astrocytes [56]. Therefore, it was conceivable to hypothesize that injury-induced Ca^{2+} signaling in rat aortic ECs was mediated by ATP liberated nearby the lesion site. Accordingly, 300 μM suramin, a broad spectrum inhibitor of ATP-sensitive P_2 receptors, reduced the peak Ca^{2+} response to injury to ≈20% of its normal value. This concentration of suramin was able to abrogate the Ca^{2+} transient induced by a maximally effective dose of ATP (100—300 μM, unpublished data from our lab). It followed that an additional mechanism was likely involved in the onset of injury-induced $[Ca^{2+}]_i$ elevation. Indeed, inhibiting gap junctions/hemichannels diminished the Ca^{2+} raise by 20% (see below).

P_2 receptors may be divided into two subgroups, P_{2X}, ATP-regulated, Ca^{2+} permeable ion channels and P_{2Y}, G-protein coupled metabotropic receptors [31]. A previous study of endothelium-dependent relaxation of rat aorta provided the evidence that both P_{2Y1} and P_{2Y2} receptors are co-expressed in rat aortic ECs [32]. This result was supported by the subsequent findings that rat aortic ECs *in situ* may produce a Ca^{2+} response to 2-MeSATP (an agonist of P_{2Y1} receptors), ADP (an agonist of P_{2Y1} and P_{2Y12} receptors; see below), UTP (an agonist of P_{2Y2} receptors) and ATP (which activates both P_{2Y1} and P_{2Y2}) [27; unpublished observations from our lab]. However, recent evidences have shown that cultured vascular ECs may express also P_{2X} and P_{2Y12} receptors [33,57]. Our effort to characterize the receptor isoforms responsible for the P_2-operated component of the peak response by means of specific inhibitors and desensitization led to the following results: (1) injury-evoked increase in $[Ca^{2+}]_i$ impinges on both P_{2X} and P_{2Y} receptors; (2) P_{2X} receptors, which were desensitized by previous preexposure α,β-MeATP, account for ≈15% of the response; (3) P_{2Y1} and P_{2Y12} receptors, which were inhibited by MRS-2179 and 2-MeSAMP, respectively, account for ≈20% each of the response; (4) P_{2Y2} receptors, which miss a specific blocker, likely account for the remaining of the P_2-sensitive component of the peak response to injury. While the ionotropic P_{2X} receptors participate to injury-induced Ca^{2+} signaling by mediating Ca^{2+} inflow into the cells, the G-protein cou-

pled P_{2Y} receptors trigger only intracellular Ca^{2+} release from IP_3Rs. The latter finding was rather surprising, since BTP-2, the most selective SOCs inhibitor, was able to fully block 300 μM ATP-induced Ca^{2+} influx into rat aortic ECs (see Fig. 4B). However, BTP-2 affected neither the peak nor the decay phase of injury-induced Ca^{2+} mobilization. In a previous study, we have demonstrated that at bulk concentration $\leq$20—50 μM ATP activates Ca^{2+} release, but not Ca^{2+} influx, due to the degrading activity of ectonucleotidases [27]. Therefore, it is conceivable that ATP liberated at the injury site undergoes breakdown too quickly to evoke any apparent Ca^{2+} influx. The strong ectonucleotidases activity of rat aorta ECs *in situ* further suggests that: (1) a quick ATP degradation might account for the short lasting and spatially restricted effect of ATP in rat aortic ECs *in situ* as compared with injury-evoked Ca^{2+} waves in CPAE [14; see below]; and (2) ATP breakdown might lead to the accumulation on cells surface of metabolic derivatives, such as ADP, AMP and adenosine, which could contribute to injury-induced Ca^{2+} increase. Unlike ADP, however, AMP elicits a Ca^{2+} response in rat aortic ECs *in situ* only at high concentrations (1 mM), while adenosine has been shown to be ineffective [27].

4.2. The injury-evoked Ca^{2+} signal: the decay phase

The initial Ca^{2+} peak induced by injury was followed by a prolonged decay phase due to Ca^{2+} influx. As aforementioned, store-operated Ca^{2+} entry does not sustain the overall signal elicited by injury, as shown by the lack of effect of BTP-2. Consistent with this evidence, Gd^{3+}, a cation able to act on a variety of Ca^{2+}-permeable channels, including SOCs [40], did not modify the decay phase of injury-elicited Ca^{2+} signal. The latter result is in contrast with what observed in cultured monolayers of CPAE, where Gd^{3+} did impair wound-induced $[Ca^{2+}]_i$ increase [52]. Additional routes for Ca^{2+} inflow into ECs might be provided by L- and T-like VGCC and MCC [19]. However, VGCC are apparently lacking in rat aortic endothelium *in situ*, while the contribution of MCC to the decay phase is ruled out by the insensitivity of the Ca^{2+} signal to Gd^{3+}, which may also inhibit stretch-sensitive channels [19]. As mentioned in Section 1, ECs may be endowed also with RACCs. In this view, the inhibitory effect of La^{3+} and Ni^{2+} (see below) might be ascribed to the block of such a Ca^{2+}-permeable membrane pathway [19]. Remarkably, neither SK&F96365, a widely employed blocker of endothelial RACCs [57], nor PLC inhibition impaired the decay phase of injury-evoked Ca^{2+} signal, thus ruling out RACCs participation. Therefore, a non-conventional Ca^{2+} pathway might be open, and it was supposed that gap junctions were involved.

The gap junction channel is made by two connexons (or hemichannels), each formed by six molecules of connexins. The two hemichannels are inserted into the plasma membrane of two neighboring cells and are assembled to form a unique aqueous channel connecting the cytoplasm of the two cells. Rat aorta ECs mainly express connexin 40 and connexin 37 [43]. It is usually thought that an uncoupled hemichannel, which sees the extracellular milieu, is closed in order to avoid cell death [49]. Strikingly, in ECs facing the injured site, a number of gap junction/hemichannel blockers (octanol, oleamide, palmitoleic acid, heptanol, and La^{3+}) and Ni^{2+} diminished the amplitude of the peak response by ≈20% and strongly impaired the following decay phase. These data are compatible with the hypothesis that the Ca^{2+} pathway involved in the response to endothelial damage is a hemichannel or a gap junction channel (it is unknown whether injury detaches the two hemichannels, which, alternatively, might remain assembled). Therefore we suggest the following model:

(a) Injury destroys a number of ECs. ECs adjacent to scraped cells have now a number of gap junction channels/hemichannels that see the extracellular medium.
(b) These gap junction channels/hemichannels assume a closed conformation, as shown by ECs capability to retain intracellular fura-2 and to exclude extracellular ethidium bromide or Lucifer Yellow, three molecules able to cross open gap junction channels [39,40]. As stated above, gap junctions/hemichannels closure is an obligatory response, in order to preserve the intracellular milieu.
(c) However, gap junction channels/hemichannels closure is not complete, allowing a prolonged Ca^{2+} influx.
(d) Hemichannels in cultured cells or expressed in *Xenopus* oocytes have been shown to strongly reduce their permeability in the presence of physiological extracellular $[Ca^{2+}]$, and to be blocked by La^{3+} and gap junction blockers [49]. The possibility that cells uncoupling is followed by a sustained Ca^{2+} influx through uncoupled gap junction channels/hemichannels has not previously been evidenced.

The present investigation has provided a mechanistic characterization of injury-evoked Ca^{2+} signals in ECs near the edge of the denuded zone *in situ*. At least four differences between our results and those obtained from cultured cells have been found: (1) the Ca^{2+} signal generated by injury in rat aortic ECs *in situ* did not propagate farther than the fourth to fifth row of cells away from the lesion site, while it traveled up to 10—12 cell rows from the wound edge in cultured CPAE [14]; (2) the $[Ca^{2+}]_i$ elevation measured in ECs *in situ* was significantly longer than in cultured monolayers (see Fig. 2A in [14]) due to prolonged Ca^{2+} influx, possibly through gap junction channels/hemichannels; (3) the Ca^{2+} response to damage was insensitive to Gd^{3+} *in situ*, but not in culture [52]; (4) suramin could not block the response to ATP in isolated aortic ECs [58].

The injury-evoked increase in $[Ca^{2+}]_i$ is central to the processes of ECs proliferation and migration by activating a number of Ca^{2+}-dependent transcription factors [52]. Moreover, an elevation in intracellular Ca^{2+} levels has been shown to result in nitric oxide (NO) production, which may promote ECs growth during wound healing [54]. The spatial proximity of the sites of Ca^{2+} release/entry to Ca^{2+}-dependent molecular targets determines the specificity of Ca^{2+} actions [59]. Our data showed that endothelial injury may engage distinct sources for Ca^{2+} mobilization, such as P_{2X} receptors, IP_3Rs and gap junction channels/hemichannels. Experiments are under way to elucidate whether these distinct Ca^{2+} permeable pathways are selectively coupled to the different Ca^{2+}-sensitive mechanisms involved in wound repair.

Acknowledgments

The work was supported by FAR (Fondo d'Ateneo per la Ricerca, University of Pavia, Italy) and CONACYT (Consejo Nacional de Ciencia y Tecnología, Mexico). Grant No. 115230 to RBR.

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